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Interactive effects of increased temperature, pCO_2 and the synthetic progestin levonorgestrel on the fitness and breeding of the amphipod *Gammarus locusta*☆

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ABSTRACT

Given the lack of knowledge regarding climate change-chemical exposure interactions, it is vital to evaluate how these two drivers jointly impact aquatic species. Thus, for the first time, we aimed at investigating the combined effects of increased temperature, pCO_2 and the synthetic progestin levonorgestrel on survival, growth, consumption rate and reproduction of the amphipod *Gammarus locusta*. For that, a full factorial design manipulating temperature [ambient temperature and warming (+4 °C)], pCO_2 [normocapnia and hypercapnia (Δ pH 0.5 units)] and the progestin levonorgestrel (LNG: L1 – 10 $ngLL^{-1}$ and L2 – 1000 $ngLL^{-1}$, control – no progestin and solvent control – vehicle ethanol (0.01%)) was implemented for 21 days. *G. locusta* was strongly negatively affected by warming, experiencing higher mortality rates (50–80%) than in any other treatments. Instead, growth rates were significantly affected by interactions of LNG with temperature and pCO_2. It was observed, in the short-term (7d) that under ambient temperature (18 °C) and hypercapnic conditions (pH 7.6), the LNG presence promoted the amphipod's growth, while in the medium-term (21d) this response was not observed. Relative consumption rates (RCRs), during the first week were higher than in the third week. Furthermore, in the first week, RCRs were negatively affected by higher temperature while in the third week, RCRs were negatively affected by acidification. Furthermore, it was observed a negative effect of higher temperature and acidification on *G. locusta* fecundity, contrarily to LNG. Concluding, the impact of increased temperature and pCO_2 was clearly more adverse for the species than exposure to the synthetic progestin, however, some interactions between the progestin and the climate factors were observed. Thus, in a future scenario of global change, the presence of LNG (and other progestins alike) may modulate to a certain level the effects of climate drivers (and vice-versa) on the gammarids fitness and reproduction.

1. Introduction

Future ocean conditions will challenge marine organisms with a variety of ecosystem-level stressors which will be exacerbated by global change (Byrne and Przeslawski, 2013; Boyd et al., 2016). Temperature and pCO_2 are among the most relevant environmental stressors that control the distribution and performance of marine species (Portner and Farrell, 2008; Kroeker et al., 2010; Byrne, 2011). As atmospheric CO_2 concentrations increase, ocean pH will tend to decline, almost 0.3–0.4 units by the year 2100, while global sea surface temperature is expected to increase 1.1–6.4 °C for the

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same period (Meehl et al., 2007). Besides these factors, organisms are exposed to a multitude of abiotic and biotic stressors (e.g. salinity, ultraviolet radiation, nutrients, chemical pollution) which can interfere with temperature and/or pCO_2 making the biological responses even more complex.

In the last years, literature regarding the combined impacts of acidification and warming has increased considerably, which allows us to better understand the complexity of responses associated to different species (Harvey et al., 2013). Besides climate change, there is a growing concern about some chemical compounds, like pharmaceuticals whose consumption has increased in the last years and tend to rise even more in a near future (Kummerer, 2009; Fent, 2015; Kay et al., 2017). Among the most critical pharmaceuticals are steroid hormones that may act as endocrine disruptor chemicals (EDCs). According to the literature, steroids are among the most mighty endocrine disrupters reaching aquatic ecosystems through excretion by humans and livestock and from their use as contraceptives (Orlando and Ellestad, 2014; Overturf et al., 2014; Fent, 2015; Kumar et al., 2015). Progestins or gestagens are a class of synthetic steroids with progestogenic activity, which have been scarcely studied as EDCs. Particularly, levonorgestrel is a synthetic progestin used as a contraceptive as well as post-coital contraception modality ("the morning after pill") that has been detected in the range of ng L^{-1} in effluents of sewage treatment plants, surface and ground waters and also in sediments (de Alda et al., 2002; Vulliet et al., 2007, 2008). Previous studies have already demonstrated its negative effects on reproduction and development of distinct aquatic species (Runnalls et al., 2013; Overturf et al., 2014). However, there is a complete lack of knowledge about the effects of climate stressors on the performance/behaviour of those steroids (and vice-versa) and consequent effects on aquatic ecosystems.

Beyond the effects of chemical and nonchemical stressors in aquatic organisms, environmental changes have the ability to modify the toxicokinetics of chemicals that consequently can have implications at organism and population level responses (Hooper et al., 2013; Di Lorenzo et al., 2015; Noyes and Lema, 2015). Interactive effects between climate change and chemical pollution are extremely complex, since on one hand, environmental variables can change the sensitivity of the organisms to the chemicals and on the other, the pollutants can modify the organisms' ability to respond to climatic conditions (Hooper et al., 2013; Noyes and Lema, 2015). Considering these facts and attending to the few works done on climate change-chemical exposure interactions (e.g. Jacobson et al., 2008; Di Lorenzo et al., 2015; Cardoso et al., 2017) it is vital to evaluate and understand the response of aquatic organisms to all those global change drivers. Crustaceans are ubiquitous in the aquatic ecosystem and are considered reliable bioindicators of contamination and environmental changes (Neuparth et al., 2002, 2014; Costa et al., 2005). Specifically, *Gammarus locusta* is a species with a strong ecological relevance and high sensitivity to contaminants, which associated to the short life cycle and ease to keep and breed in the laboratory, makes it a good model to assess the effects of contaminants along its life cycle (Costa et al., 2005). Therefore, in this study, we aimed to test the conjugation effects of temperature, pCO_2 and levonorgestrel stressors to the amphipod *Gammarus locusta* in order to contribute to the understanding of the vulnerability of this species to environmental changes. Here, we applied an integrative approach, considering multiple key endpoints at individual level, such as survival, growth rate, consumption rate and reproduction.

2. Materials and methods

2.1. Pharmaceutical

The standard levonorgestrel (LNG; CAS 797-63-7; purity = 99%) was purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions were prepared with analytical ethanol (CAS 64-17-5; purity ≥ 99.9%) supplied by Merck and stored in dark at −20 °C.

2.2. Amphipod collection and acclimation

The amphipod *Gammarus locusta* is a marine epibenthic crustacean that feeds mainly on green macroalgae (e.g. *Ulva* spp). and presents a vast geographical distribution along the European coastal systems, including the Portuguese coast (Costa and Costa, 2000). The individuals used in the experiment were obtained from a permanent laboratory culture system at CIIMAR facilities (Portugal) in which the original specimens were collected from the south margin of Sado estuary, Portugal (Neuparth et al., 2002).

G. locusta individuals were separated by size and sub-adult individuals (with approximately 3–4 weeks) were selected for the experiment. During acclimation period (7 days), animals were fed with *Ulva* spp. on an *ad-libitum* basis and maintained in a semi static system whereby 100% of the water was changed twice a week. Tanks were filled with a sand layer (1 cm) and pebbles in order to mimic the natural environment. Photoperiod was set to 18 h light: 6 h dark to simulate summer conditions. The organisms were acclimated under the ambient temperature and normocapnia (18 °C, pH 8.1) and salinity 33–35. These conditions corresponded to the mean sea surface temperature (SST) and pH at the Sado estuary.

2.3. Experimental design

The experimental set-up followed a full factorial design manipulating temperature [ambient temperature and warming (+4 °C)], pCO_2 [normocapnia (pCO_2 = 400 μatm) and hypercapnia (pCO_2 = 1600 μatm; Δ pH 0.5 units)] and the progestin levonorgestrel (LNG: L1 − 10 ngLL^{-1} and L2 − 1000 ngLL^{-1}, control − no progestin and solvent control − vehicle ethanol (0.01%)) in a total of 16 treatments (Table 1). During exposure, the amphipods were maintained at the same salinity as during acclimation (33–35). In addition, the organisms were gradually exposed to the increase of temperature (1 °C/day) until reaching the highest temperature (i.e. 22 °C).

In order to avoid the interdependence or non-randomly interspersed treatment replicates that is frequently common in ocean acidification studies, we have implemented one of the experimental models suggested by Cornwall and Hurd (2015). Therefore,

Table 1
Description of the different treatments to which *G. locusta* were exposed. C − control, SC − solvent control, L1 - LNG (10 ng L^{-1}) and L2 - LNG (1000 ng L^{-1}).

Treatments	Condition
T1	18 °C, pH 8.1, C
T2	18 °C, pH 8.1, SC
T3	18 °C, pH 8.1, L1
T4	18 °C, pH 8.1, L2
T5	18 °C, pH 7.6, C
T6	18 °C, pH 7.6, SC
T7	18 °C, pH 7.6, L1
T8	18 °C, pH 7.6, L2
T9	22 °C, pH 8.1, C
T10	22 °C, pH 8.1, SC
T11	22 °C, pH 8.1, L1
T12	22 °C, pH 8.1, L2
T13	22 °C, pH 7.6, C
T14	22 °C, pH 7.6, SC
T15	22 °C, pH 7.6, L1
T16	22 °C, pH 7.6, L2

a model with different tank types (i.e. storage tank, mixing tank, and experimental tank) was applied (see in detail Cornwall and Hurd (2015)). The continuous flow-through system was divided in four levels. The first level corresponded to the reservoir tank (i.e. 1 seawater tank), the second fitted to the CO_2 mixing tanks (i.e. 2 acidic and 2 non-acidic), the third corresponded to the LNG mixing flasks (total of 16) and finally the fourth level corresponded to the experimental units level (total of 96 units) (Fig. 1).

Water flow through the different tanks was maintained by gravity whereby water level in each tank was controlled by a float valve that closed once water level reached the required volume. The same mechanism was employed to control water in CO_2 mixing tanks (4) and contaminant mixing flasks (16) in the second and third levels, respectively. Seawater from this reservoir was then directed to the second level, which comprised the CO_2 mixing tanks (50L). CO_3^{2-} chemistry in the acidic tanks was manipulated by diffusing pure CO_2 from a gas tank. CO_2 gas flow was regulated through a pH stat system (Aqua Medic® AT Control-SW, version 9.0). This was made possible as the AT Control automatically opened or closed a solenoid valve when pH readings in the acidic tanks deviated from the predetermined set points by 0.1 pH units. pH readings were recorded through pH electrode sensors. CO_2 mixing tanks were maintained with aeration to facilitate CO_2 diffusion. Following acidification, seawater from both acidic (Ac) and non-acidic (NAc) tanks was directed to the contaminant mixing flasks. Each Ac and NAc tank continuously filled four contaminant glass flasks (5L each), where coloured codes were used to distinguish treatments into LNG contaminant flaks, solvent control and control flasks.

The flow rate from the contaminant mixing flasks (third level) to the respective experimental units was ensured by a valve mechanism and maintained at 0.009 L min^{-1}. Experimental units assigned in different treatments were maintained at different temperatures in water baths (30L), in order to keep the temperatures more stable during the experiment. Temperature within the water baths was also regulated by the AT Control, which automatically heated the tanks whenever temperatures deviated from predetermined set points by 0.1 °C. Experimental units were illuminated with artificial light apparatus suitable for marine set ups. Photosynthetic Active Radiation (PAR) was measured with a universal light meter (ULM – 500; WALZ) and maintained at approximately 14 μmol photons m^{-2} s^{-1}.

Each experimental unit was continuously aerated and concurrently, water level in each unit was maintained at 0.55 L through a dewatering tube connected to an external flask (0.80 L), which was working on the principle of communicating vessels. From these external flasks, contaminated water was directed to a contamination tank (20 L) and with a submersible pump (3000 L h^{-1}) water was pumped continuously to the decontamination chamber (120 L). Wastewater was disposed through an activated-charcoal filter before being eliminated.

Amphipods were exposed to 16 different treatments indicated in Table 1. Each treatment included 6 replicates (glass cups of 650 mL each, diameter of 8 cm) that were distributed randomly by 6 water baths. Each replicate included 14 individuals of *G. locusta*, in a total of 1344 individuals. Four of these (not separated by sex) were individualized in small containers inside the glass cups in order to evaluate individual survival, growth, condition index and consumption rates. The remaining ten individuals were maintained free in the cups, for reproductive purpose. Only these were separated by sex, so, three replicates contained males and the other three contained the females to avoid mating during exposure period, which could influence the results of reproduction, posteriorly.

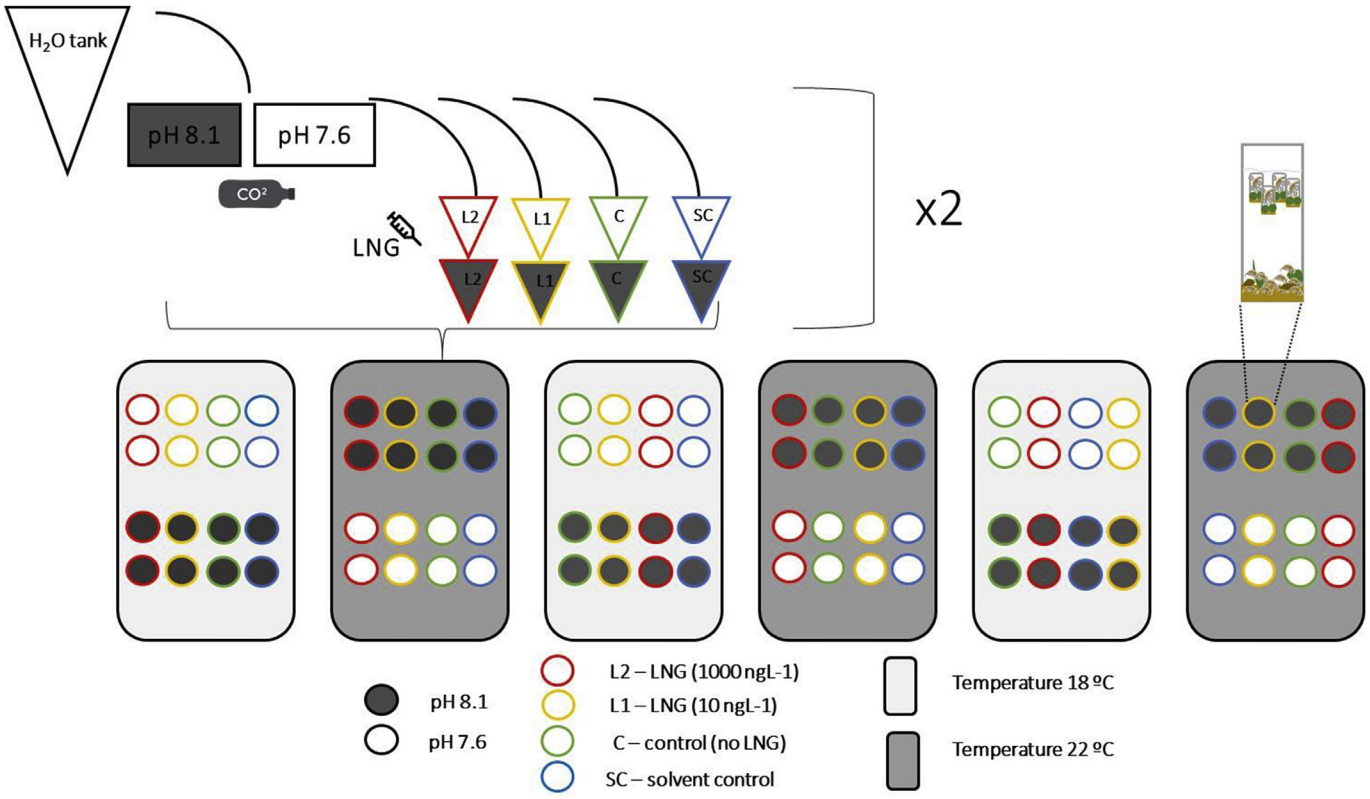

Fig. 1. A schematic representation of the experimental set-up in which are represented the 4 levels of organization (i.e. first level - sweater tank (1), second level - CO_2 mixing flasks (4), third level – contaminant mixing flasks (16) and fourth level – experimental units (96)). A detailed scheme of each experimental unit (i.e. glass cup) is also represented.

To simulate the natural environment, inside the cups a thin layer of sand (≈1 cm) and *Ulva* spp (2 discs; 0.0708$g_{w/w}$ ± 0.102 per individual) were added. The sand was previously burnt to eliminate any trace compounds. *Ulva* discs were replaced thrice a week.

The experiment was divided in two phases: 1) pre-mating and 2) post-mating. During pre-mating, the organisms were exposed to LNG three times a day, during 21 days, to simulate episodic discharges from a contamination source. So, at each time of the day, LNG was injected directly into the contaminant mixing tanks and the flow rate passed directly to the experimental units (i.e. glass cups). In the post-mating phase, after 21 days exposure and all the individualized organisms have been sampled, couples of *G. locusta* were arranged based on the individuals that were maintained free in the cups and subject to the treatments. So, one couple was allocated to each replicate (i.e. glass cup) for mating and breeding, without LNG exposure (see more details of this phase in section 2.7). Besides the individuals that were used to form the couple, remaining ones were sampled for other purposes (e.g. genotoxicity studies) that will be addressed in other manuscript.

Survival was checked at days 7, 15 and 21, and the individualized organisms sampled at day 21 were examined for growth and condition estimates.

During the experimental period, water temperature and pH were controlled and varied in the range of ambient temperature: 18.230 °C ± 0.447, warming temperature: 21.791 °C ± 0.587, normocapnia: 8.057 pH ± 0.0562 and hypercapnia: 7.520 pH ± 0.255.

2.4. Amphipod length and weight

Amphipods that were kept isolated for growth estimates were divided in two groups. Half of them were measured and weighted at day 1 and day 7. The others were measured and weighted at day 1 and then at day 21. Besides the endpoints assessed in the present work, there was another objective of collecting individuals for histopathological studies (results to be addressed in other work) at days 7 and 21 that is why we have followed 2 different groups of individuals. Amphipods were previously photographed individually using the Olympus cellˆB (analySIS image processor version 5.1) program. The program, ImageJ (version 1.50i) (Rasband 2016) was then used to determine the individual Metasomatic Length (ML) to the nearest 0.1 mm, following the same procedures as Neuparth et al. (2002). Since it is difficult to determine total length (TL) of the animal, due to its lateral resting position, alternatively ML was measured (Costa and Costa, 1999). ML is defined as the distance between the anterior end of the rostrum and the posterior end of the last metasomatic segment (Neuparth et al., 2002). The relationship between TL and ML had been previously determined by Costa and Costa (1999) and is represented by the following linear regression equation:

$$TL = -0.153 + 1.218^{*}(ML)$$

Hence, TL of individuals along the exposure phase was determined using the above linear regression. In addition, at each time the amphipods were weighed (whole weight) using a AND GR-200 analytical balance.

2.5. Amphipod condition index

Amphipods length and wet weight data were used to determine the fitness condition of organisms along the exposure period. In this case, the Fulton Condition Index (K) was applied and is expressed by the following (Ricker, 1975):

$$K = W/TL^3 \text{ x } 100$$

Where: W = mean weight and TL^3 = mean total length.

This index is typically used for fishes (Rosa et al., 2014) but it has also been applied for crustaceans (Enin, 1994; Moreira et al., 2015).

2.6. Consumption rate

Relative consumption rates (RCRs) were evaluated twice during the experimental period. The first assay was conducted during the first week and the final one in the third week of exposure. The individuals selected for consumption (the individualized ones) were starved for 24 h prior to the consumption experiment that run for 24 h.

Using the equation adopted by Gutow et al. (2014), amphipod consumption (C) was calculated as C = Wi * (Cf/Ci) − Wf; where, Wi and Wf are the initial and the final WW of the *Ulva* sp. pieces, respectively, and Ci and Cf are the equivalent WW of the control pieces (biogenic controls). Relative consumption rates (RCRs, g *Ulva* ww g^{-1} individual ww day^{-1}) were calculated as C/(WM_f x t), where C is the *Ulva* sp. consumed for each time interval (1d) and WM_f is the final wet mass of individuals for each time interval.

2.7. Reproductive traits

The reproductive behaviour of *G. locusta* involves a precopulatory guarding phase in which the male holds and carries the female. This ensures that insemination can occur as soon as the female moults and is ready to release eggs into the marsupium (Maranhão and Marques, 2003). During this phase, couples were observed daily until females were separated from males and observed to be ovigerous. Embryonic development time was measured as the period from oviposition to release of juveniles from the brood pouch of females (Maranhão and Marques (2003). Hence, ovigerous females were observed daily until the marsupium was noticed to be completely empty. As soon as females were observed with an empty marsupium, experimental units were filtered through 500 μm and 250 μm sieves to separate juveniles from females.

Fecundity of *G. locusta* was accounted as the number of neonates released per female in each replicate. Following the separation from females, neonates were straight away sacrificed and stored away in 70% ethanol for counting. Juveniles were counted using stereomicroscope (Leica EZ4).

2.8. LNG analysis from water samples

2.8.1. Water sampling

Water samples (2 L, n = 3 replicate per treatment) from all exposure groups were collected in amber flasks just after the LNG addition (T_0) and before the water renewal (T_{24}). During sampling, all bottles were rinsed two or three times before the collection of the water samples, which were filtrated, to eliminate particulate matter and other suspended solids, through a 0.45 μm GF/C glass fiber filter, acquired from Millipore (Ireland). After this procedure, each filter was washed with small amounts of CH_3OH that were added to the latter filtrate. Then, water samples were acidified with H_2SO_4 to pH 2 to prevent biodegradation and kept at ca. 4 °C, before the preconcentration step which occurred within a maximum of 48 h.

2.8.2. Solid-phase extraction

LNG was extracted from water samples, by solid-phase extraction (SPE) using 200 mg OASIS HLB cartridges purchased from Waters Corporation (Milford, MA, USA) adapted in an off-line SPE vacuum extraction device (Waters). Prior to use, the cartridges were sequentially washed with 13 mL of methanol: dicloromethane (CH_2Cl_2:CH_3OH, 50:50, v/v), 6 mL of CH_3OH and 25 mL of ultrapure

Milli-Q water, following a method initially developed to extract phenolic compounds and steroids in water samples. Water samples were loaded onto SPE cartridges, at a constant flow rate of 5–7 mL min^{-1} followed by a washing step with 13 mL of ultrapure Milli-Q water and 500 μL of methanol. Cartridges were dried under vacuum for 30 min and then mounted with 1 g Sep-Pak silica cartridges, previously conditioned with 10 mL of $CH_3OH:CH_2Cl_2$ (50:50, v/v), at 1 mL min^{-1}. The resulting extracts were evaporated to dryness in a heating block, at 40 °C under a gentle stream of nitrogen, and reconstituted in 20 μL of acetonitrile (C_2H_3N) before analysis. This method successfully allowed the recovery of LNG (103.7%) with a precision of 6.6%.

2.8.3. Instrumental and main methodological characteristics

The quantitative analysis was performed by a Thermo Accela™ high-speed chromatographic system (U-HPLC), equipped with a PDA, optimized for the detection of high speed chromatographic separations, a quaternary pump, an autosampler and oven all from Thermo Scientific. The analytic column was the Acquity UPLC BEH C_{18} column (1.7 μm, 2.1 × 100 mm, no. 186002352) from Waters™.

The quantitative analysis in this system was settled for LNG. So, the volume of the injected sample was 5 μL and the mobile phase was a mixture of CH_3CN, HPLC grade (≥99.93%), and ultrapure Milli-Q water (50:50, v/v), previously filtered and degassed. The elution mode was isocratic, at a constant flow rate of 350 μL min^{-1}, and the column oven was settled for 40 °C. All quality control parameters were performed taking in consideration the International Conference of Harmonization rules (1996). Here, the retention time of LNG was 1.1 min (RSD< 1%) and only the peak showing purity test higher than 99% (value calculated automatically by the PDA software) was considered for the quantitative analysis. Using this protocol, the limits of method detection (LOD) and quantification (LOQ) for LNG were 1.2 ng L^{-1} and 4.0 ng L^{-1}, respectively.

2.9. Data analysis

Experimental results were examined using linear models. Initially, models for all the responses were constructed including fixed factors and random effects (i.e. linear mixed models, LMM). However, when random effects did not improve the model fit, we applied the parsimonious principle, removed those terms and used generalized linear models (GLM).

Hence, to examine the effects of the treatments on the survival of *Gammarus locusta* at the end of the experiment we used generalized linear models assuming a Binomial distribution of the data. To visualize the survival curves we used the function *ggsurvplot* (in *Survminer* R package) to produce the survival curves for all the treatments which are represented as survival percentages. The model included as predictors the three experimental factors: temperature, pCO_2 and LNG concentrations. Analogous linear models were constructed to examine Fulton's K, consumption rates, reproduction, fecundity and embryonic development, but this time data were assumed to have a Gaussian distribution. Growth rate of the amphipods was examined after days 7 and 21 experimental days. To facilitate the analyses and prevent complex interactions the analyses of both periods were done independently. This time we used linear mixed models including as fixed predictors the temperature, pCO_2 and LNG concentration and tank (i.e. temperature water bath) as a random factor nested in temperature.

In all the models, significant predictors were selected from the full models by removing sequentially those predictors of higher order and with the higher p values, and comparing the reduced model with the original one using analyses of variance (ANOVA).

When significant interactions were found, treatments were compared using p-adjusted Tukey tests ($p < 0.05$). It was used Lsmeans package for R to perform these tests *a posteriori* (Lenth, 2016).

All the statistical analyses were run in R environment (R Core Team, 2016). Linear mixed models were run using nlme package (Pinheiro et al., 2017). Assumptions for the linear models were checked by examining the residual plots. In the case of response variables with Gaussian probability distribution and when required, data were log transformed to reduce heteroscedasticity.

3. Results

3.1. Levonorgestrel in water

Measured concentrations of LNG at 30 min (T_0) and 90mn (T_{90}) immediately after the first injection are indicated in Table 2. At T_0 the measured concentrations were close to the nominal values, however, at T_{90} the concentrations decreased considerably, reaching approximately half of the nominal values. The concentrations in the control and solvent control replicates were below the limit of

Table 2
Nominal and measured concentrations of levonorgestrel (ng L^{-1}) in waters during 21 days exposure. The water was sampled 30 min after the first injection (T_0) and 90 min later (T_{90}). The values are expressed as the means ± SE (n = 3).

Treatments		Nominal	T_0	T_{90}
18 °C pH 8.1	C	–	<1.2	<1.2
	SC	–	<1.2	<1.2
	L1	10	11.4 ± 0.0	<1.2
	L2	1000	928.2 ± 31.6	406.9 ± 27.6
18 °C pH 7.6	C	–	<1.2	<1.2
	SC	–	<1.2	<1.2
	L1	10	11.4	<1.2
	L2	1000	915.1 ± 60.1	385.2 ± 23.8
22 °C pH 8.1	C	–	<1.2	<1.2
	SC	–	<1.2	<1.2
	L1	10	11.9 ± 0.4	<1.2
	L2	1000	929.6 ± 31.6	393.7 ± 50.2
22 °C pH 7.6	C	–	<1.2	<1.2
	SC	–	<1.2	<1.2
	L1	10	11.9 ± 0.4	4.6 ± 0.1
	L2	1000	931.4 ± 60.1	354.2 ± 20.0

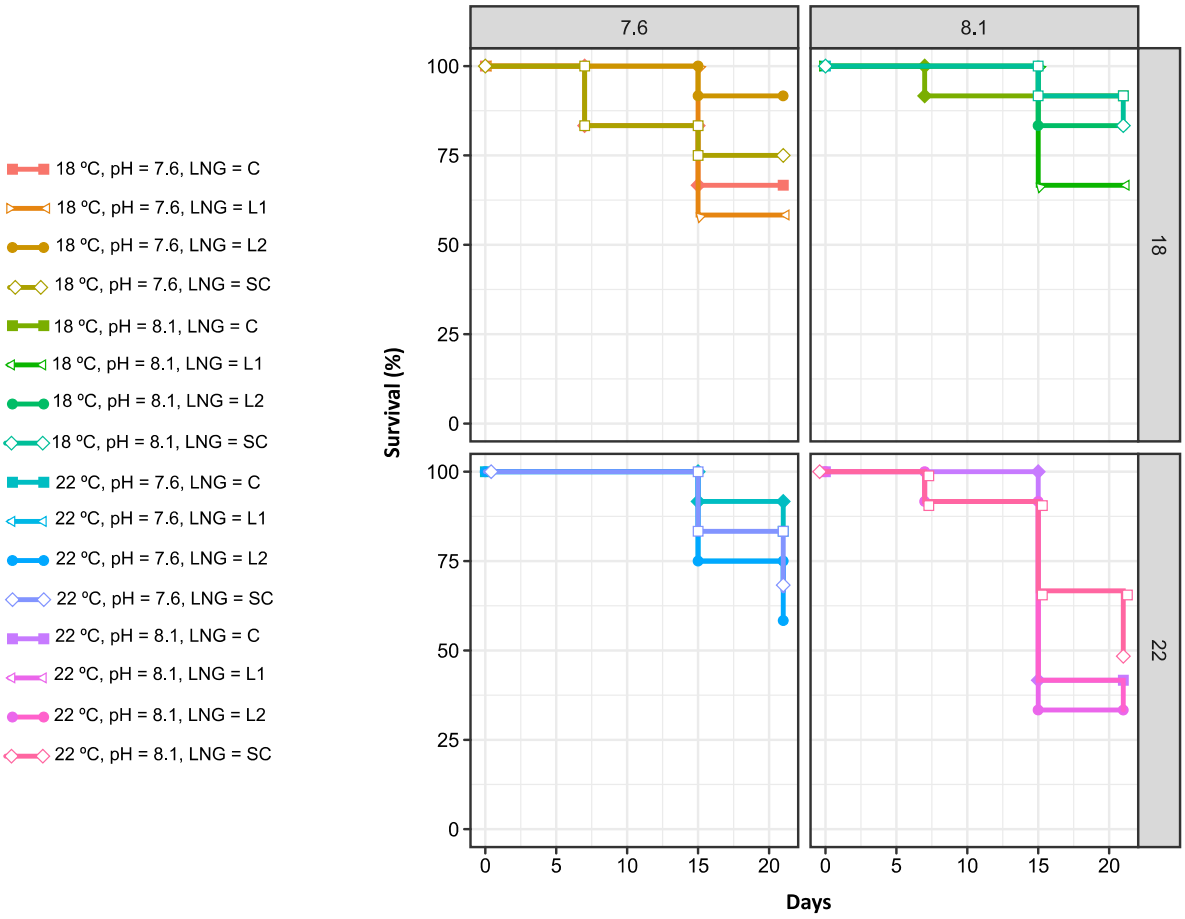

Fig. 2. Survival (%) of *G. locusta* exposed to different combinations of temperature (18 °C and 22 °C) and pH (7.6 and 8.1) under distinct LNG concentrations (n = 12 per treatment). C – control, SC – solvent control, L1 - LNG (10 ng L^{-1}) and L2 - LNG (1000 ng L^{-1}).

detection (1.2 ng L^{-1}).

3.2. Survival and Fulton's K

Regarding survival, warming condition (22 °C pH 8.1) was the one that caused the highest negative impact on the amphipods, reaching the lowest survival values (30–50%) at day 21. On the opposite, ambient temperature under normocapnia (18 °C pH 8.1) witnessed the highest survival rates (70–90%). The acidification condition (18 °C pH 7.6), as well as, the warming plus acidification scenario (22 °C pH 7.6) presented an intermediate response (60–90% survival). At day 7 the survival values were similar for most combinations of temperature and pH, reaching 80–90%, except for 22 °C pH 7.6 that showed 100% survival. The latter was the only treatment where signs of mortality occurred later (at day 15) and highest survival rates were observed at this time (Fig. 2).

Still as to survival, no significant effects of the hormone LNG, for each combination of temperature *vs* pH were observed (GLM model, $p > 0.05$). Comparing all the treatments, significant interactions between temperature and pH were detected for times 15 and 21 (GLM model, $p < 0.05$). Warming condition was significantly different from the ambient ($p = 0.0004$), acidification ($p = 0.006$) and warming plus acidification scenario ($p = 0.035$).

Regarding Fulton's K, it was observed a significant decline (GLM model, $F_{(1,127)} = 5.66$, $p = 0.0189$) in those individuals exposed to higher temperature compared to the ambient one (Fig. 3).

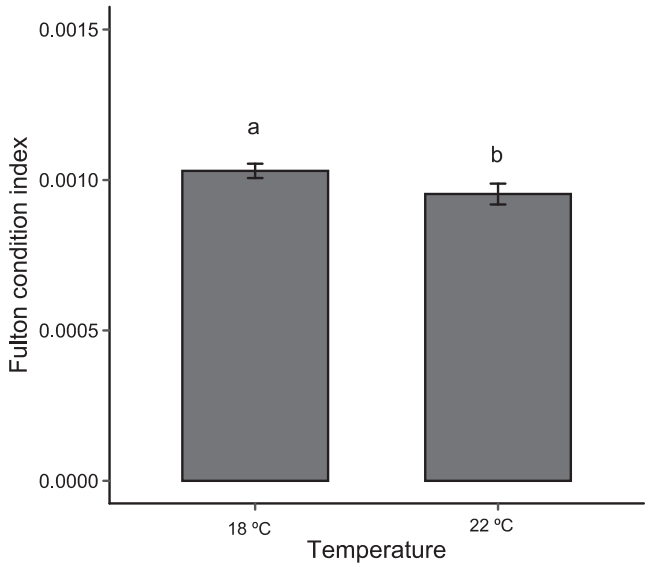

Fig. 3. Fulton condition index of *G. locusta* exposed to different treatments (n = 12 per treatment). Values represent mean (±SE). Data is displayed relative to significant factors. Different letters indicate significant differences among treatments ($p < 0.05$).

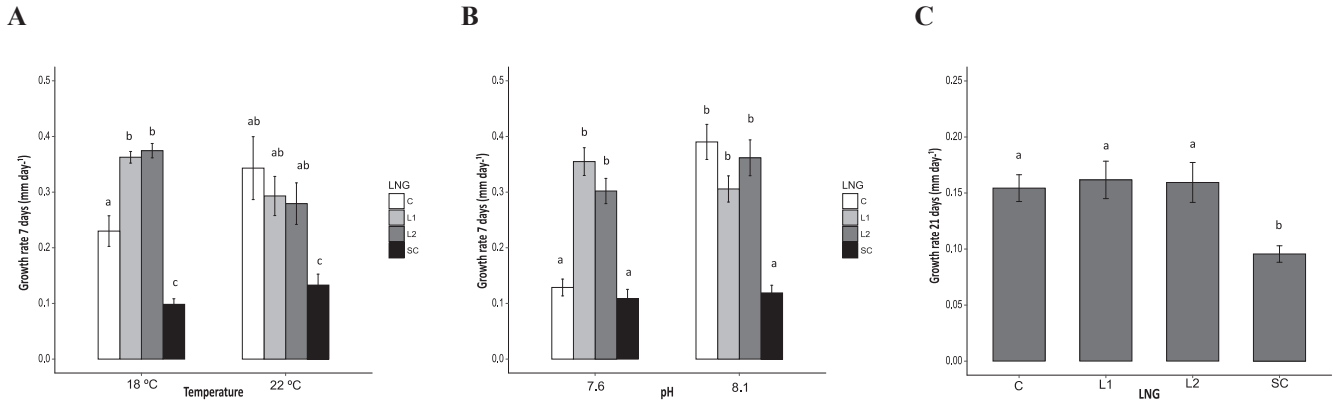

Fig. 4. Growth rates of *G. locusta* for the first 7 days (A, B) and the entire period (21 days) (C). Values represent mean (±SE) (n = 12 per treatment). Data is displayed relative to significant factors. Different letters indicate significant differences among treatments ($p < 0.05$).

3.3. Growth rates

Growth rates were estimated at two different periods of the experiment; during the first week and for the entire exposure period (21d). According to our results, in the first week, growth rates were approximately twice as high as for the entire period. Thus, in the first week, significant interactions were observed between temperature and LNG concentration (LMM model, $F_{(3,172)} = 3.556$, $p = 0.0156$) (Fig. 4A), and between pH and LNG concentration (LMM model, $F_{(3,172)} = 11.507$, $p < 0.0001$) (Fig. 4B). For the lowest temperature, it was observed a significant growth promotion effect of the hormone, which was not observed in a warming condition. On the other hand, acidic waters had a significant negative effect on the growth rates of control individuals. Once again, the presence of LNG stimulated growth in more adverse conditions. In all the cases, individuals from the solvent control presented significantly lower values than the remaining treatments.

Considering the entire experimental period, only a significant effect of the hormone LNG was detected (LMM model, $F_{(3,155)} = 7.355$, $p = 0.0001$) (Fig. 4C) and individuals from the solvent control presented significantly lower growth rates ($p < 0.05$) than the remaining treatments.

3.4. Relative consumption rates

Consumption rates were also evaluated in two different periods of the experiment. During the first week, higher consumption rates (more than double) were observed compared to the third week exposure. In addition, a significant interaction between temperature and LNG was observed (GLM model, $F_{(3,76)} = 7.267$, $p = 0.0002$) and significantly higher RCRs were observed for the control at ambient temperature compared to the other treatments and also in relation to those exposed to warming (Fig. 5A). For the third week, only significant effects of pH ($F_{(1,57)} = 8.835$, $p = 0.0043$) and LNG ($F_{(3,57)} = 4.920$, $p = 0.0041$) were observed. No significant interactions among factors were detected. In the third week, RCRs were approximately half of the first week and acidification seemed to have a negative effect (Fig. 5B). On the other hand, only individuals exposed to the lowest LNG concentration (L1) showed significantly higher RCRs than the solvent control (Fig. 5C).

3.5. Reproduction

Individuals from all the experimental treatments have reproduced however, in some of them the percentage was lower than 100%. This fact was more evident in more acidic conditions, especially at ambient temperature and also under higher LNG concentrations (Fig. 6).

3.5.1. Fecundity

The number of newborns was significantly influenced by the three main factors: temperature ($F_{(1,47)} = 8.457$, $p = 0.0055$), pH

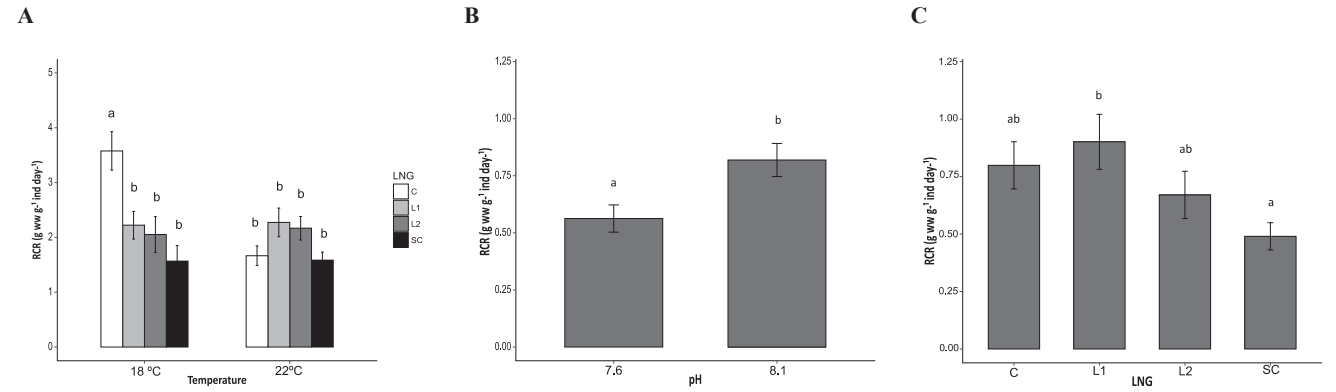

Fig. 5. Relative consumption rates (RCR) of *G. locusta* for the first week (A) and third week of exposure (B, C). Values represent mean (±SE) (n = 6 per treatment). Data is displayed relative to significant factors. Different letters indicate significant differences among treatments ($p < 0.05$).

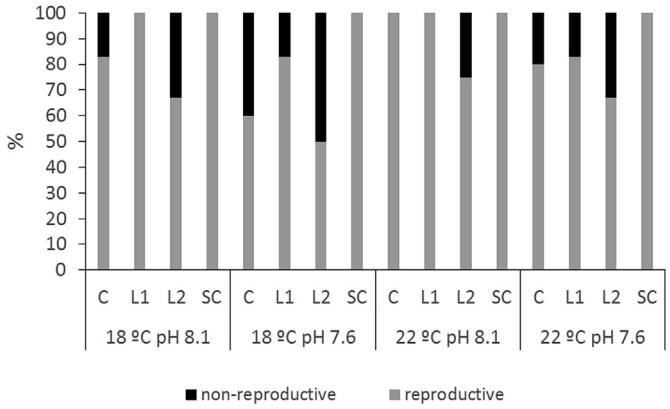

Fig. 6. Percentage of reproductive and non-reproductive individuals of *G. locusta* exposed to the different treatments.

($F_{(1,47)}$ = 5.372, p = 0.025) and LNG exposure ($F_{(3,47)}$ = 7.263, p = 0.0004). Warming and acidification conditions affected negatively the fecundity of the amphipod *G. locusta* (Fig. 7A and B). Regarding the LNG exposure, it was observed a significant increase in the fecundity of individuals exposed to the solvent control than under the remaining treatments (Fig. 7C).

3.5.2. Embryonic development

For the embryonic development, a significant interaction between temperature and LNG concentration was detected ($F_{(3,52)}$ = 0.044, p = 0.0436). Thus, a significant faster development was observed for the control treatments exposed to warming conditions. The presence of LNG at higher temperature seems to override the accelerating effect of temperature (Fig. 8).

4. Discussion

Current concerns about climate change have led to a great body of literature on the evaluation of the impact of single climate stressors (e.g. temperature and pCO_2) on marine biota (e.g. Kurihara et al., 2004, 2008; Portner and Farrell, 2008, Hauton et al., 2009; O'Connor 2009, Talmage and Gobler, 2009; Kroeker et al., 2010; Whiteley, 2011; Miranda et al., 2013; Appelhans et al., 2014). However, much less studies have been settled to assess the combined effects of both stressors (e.g. Byrne and Przeslawski, 2013; Poore et al., 2013; Kroeker et al., 2014; Rosa et al., 2014; Cardoso et al., 2017b). Even less works are available concerning the integration of climate change and chemical exposure (e.g. Jacobson et al., 2008; Di Lorenzo et al., 2015; Cardoso et al., 2017). The present study breakthrough the state of the art and allows us to understand a little bit more about the interactive effects of global change and emergent pollutants on marine aquatic life.

According to our findings, the amphipod *G. locusta* was strongly negatively affected by warming exposure (+4 °C), experiencing higher mortality rates (50–80%) than in any other treatments. Nonetheless, under the combined effects of increased temperature and pCO_2 the mortality was more reduced (10–40%). Acidification *per se*, also affected the amphipod, but in a lesser extent (10–30%). These results are corroborated by Poore et al. (2013) that observed that juveniles of amphipod *Peramphitoe parmerong* suffered a reduction in survival, on average, by 55% at day 7 and 76% at day 14 at + 3 °C. Temperature is undoubtedly, one of the most important factors affecting organisms (namely crustaceans) biological processes, like growth, reproduction, developmental rate, etc (Cifoni et al., 2017). In addition, species sensitivity to warming can vary according to life stage and life history. For some species, early developmental phases revealed to be more sensitive to temperature than adults (Byrne and Przeslawski, 2013). This could be applied to our data, since *G. locusta* used for the experiment were quite young (4–5 weeks), despite having sex differentiation, already. Reduced pH is also recognised to have negative effects on growth and survival (Byrne, 2011; Kroeker et al., 2013). According to Kroeker et al. (2013), survival and calcification were the responses most affected by acidification in molluscs, with 27% declines in both responses. Also, Talmage and Gobler (2009) observed a survival reduction of *Crassostrea virginica* larvae at pH 7.5. The hormone levonorgestrel (LNG) did not produce significant effects or interactions with the abiotic factors on the amphipod survival. However, growth rates were significantly affected by the interactions of LNG with temperature and pCO_2. So, it was observed that under ambient temperature (18 °C) and hypercapnic conditions (pH 7.6), the presence of LNG promoted the growth of the amphipod. However, under warming and normocapnia which corresponded to more favourable conditions for growth, the effect of the hormone was diluted. According to Fent (2015), synthetic progestins can be used in some countries (i.e. US and China) as growth promoters in livestock, despite the underlying mechanisms of action of progestins (and progesterone) on skeletal muscle dynamics remain controversial, even in humans (Sissom et al., 2006;

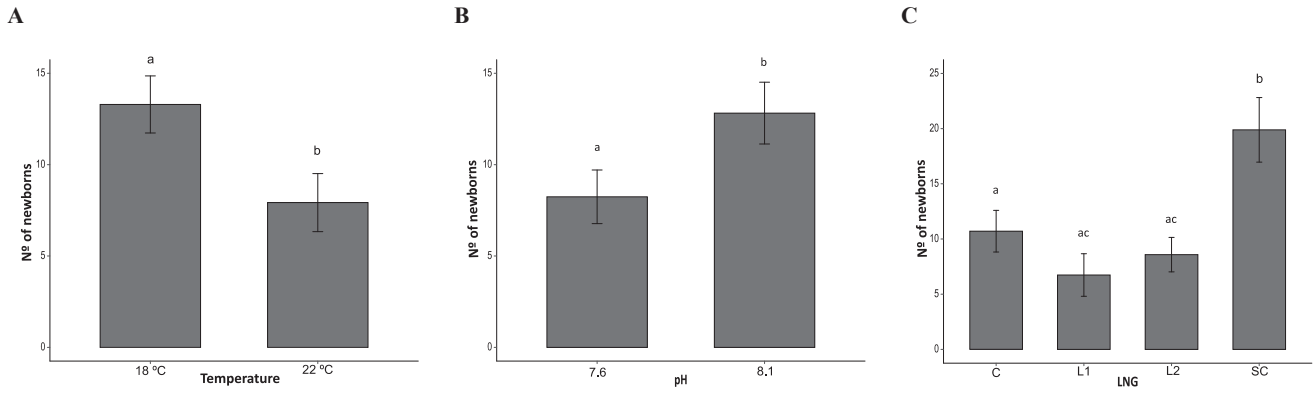

Fig. 7. Total number of newborns released by female *G. locusta* during the post-reproduction phase exposed to the different treatments. A) temperature effect; B) pH effect; C) LNG effect. Values represent mean (±SE) (n = 6 replicates per treatment). Data is displayed relative to significant factors. Different letters indicate significant differences among treatments (p < 0.05).

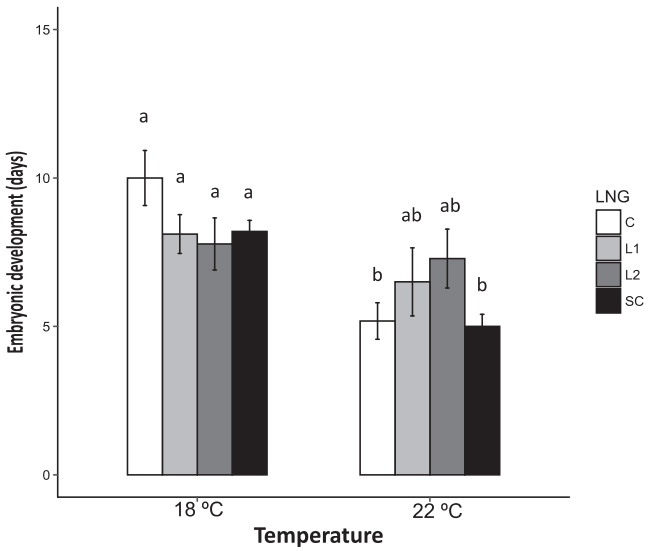

Fig. 8. Embryonic development time (days) for the *G. locusta* exposed to the different treatments. Values represent mean (±SE) (n = 6 replicates per treatment). Data is displayed relative to significant factors. Different letters indicate significant differences among treatments ($p < 0.05$).

Lopez et al., 2013; Martin and Elliott-Sale, 2016). To our knowledge, studies about the effects of progestins in aquatic organisms are still premature, especially for invertebrates' species. Most of the existent information focus on effects at fish reproduction level (see, Orlando and Ellestad, 2014; Fent, 2015; Kumar et al., 2015). A study of Overturf and Huggett (2015) on embryos of fathead minnows revealed a decline on their growth when exposed to 125 ng L^{-1} LNG. This effect was contrary to our results, however, they correspond to largely different (vertebrate *versus* invertebrate) species and in distinct developmental stages, which should influence the response.

In addition, during the first week exposure, amphipods also revealed to be sensitive to acidification, since their growth rates (at controls) were strongly affected under acidic conditions (75% reductions). Kroeker et al. (2013) revealed that all calcified taxa studied showed similar magnitude mean reductions in growth (9–17% reductions). Other previous studies, revealed that reduced pH often has a negative effect on growth (Byrne, 2011; references therein, Whiteley, 2011; Kroeker et al., 2014). Ocean acidification is energetically costly for all species increasing metabolic costs and therefore could result in slower growth rates (Kroeker et al., 2014). On the opposite, Hauton et al. (2009) did not find any effects of reduced pH on the growth rates of *G. locusta*. Our data are thus in line with the notion that depending on the taxa (calcifying or non-calcifying) and development stage, some species can be more affected than others (see in detail Byrne, 2011; Byrne and Przeslawski, 2013).

On the other hand, based on our findings, the estimated growth rates for the entire experimental period had different responses. While the above mentioned significant growth promotion effect of the hormone was observed, organisms exposed to the solvent control presented always lower growth rates than other treatments. The negative effect of the solvent can be associated to an indirect mechanism, such as solvent-stimulation of microbial growth, resulting in decreased dissolved oxygen concentrations, which can cause growth suppression (Hutchinson et al., 2006). However, if this happened, such "solvent effect" was not noted in the LNG exposed groups, being certainly overrun by the progestin growth promotion effect.

Regarding RCR, in the first week, significant higher values were observed compared to the third week. This could be related to the fact that younger individuals could have higher food needs to compensate the investment in growth than older ones. According to our results, lower RCR were observed in the controls at elevated temperature. For predator-prey interactions, metabolic theory predicts increased consumption rates with increased temperature (O'Connor, 2009). However, this is valid until a lethal threshold is reached. In the present study the highest temperature (22 °C) seems to be lethal for the amphipod *G. locusta*, which can also justify the results obtained for the consumption. The impacts caused by increasing temperatures seem to outweigh those associated to lower pH, as expected due to the dominant influence of temperature on ecological and physiological processes (Poore et al., 2013; Sampaio et al., 2017). The presence of LNG seems to negatively affect the consumption rates at lowest temperature. On the third week of exposure, gammaridae were mainly affected by both the acidification and the LNG. If some predator species can increase predation rates as a response to increased energetic demands due to the physiological effects of acidification, others can have an opposite behaviour (Kroeker et al., 2014). Acidification can also result in metabolic depression of some predators due to their inability to regulate intracellular pH. This could lead to decreased feeding rates, like observed in Appelhans et al. (2014). To our knowledge there are yet no references in the literature regarding possible effects of LNG or other steroid hormone on feeding rates of aquatic organisms.

At reproduction level, higher temperature caused a negative effect on *G. locusta* reproduction. Previous studies performed in other groups of invertebrates (eg. molluscs, polychaetes and echinoderms) have demonstrated that near-future upper warming scenarios of about 4-6 °C would not impair fertilization of those species (Byrne, 2011). There can be even increases on fertilization success, that could be due to stimulation of sperm metabolism, facilitation of acrosome reaction and increased sperm swimming speed (Byrne, 2011 and references therein). However, another study by Jacobson et al. (2008) concluded that elevated temperature impaired sexual maturation in males and females of the amphipod *Monoporeia affinis*, lowered the number of fertilised females, reduced fecundity and altered embryogenesis. These results corroborate the responses found in the present study for *G. locusta*. This is, also, in agreement with the results described in the literature for fishes and other vertebrates, in which it is demonstrated that high water temperature can impair gonadal development and block spawning (Elisio et al., 2012; Miranda et al., 2013). Unfortunately, we were unable to do the histopathological study of the gonads to prove this idea, but several studies on fishes support this hypothesis (Gillet et al., 2011; Pankhurst and Munday, 2011; Elisio et al., 2012).

On the other hand, hypercapnia also caused negative effects on *G. locusta* reproduction. Reports about the reproductive effects of ocean acidification on crustaceans are very limited and the responses are quite different depending on the species (Whiteley, 2011). Previous works have already demonstrated general negative effects of acidification on reproduction of invertebrates (Kroeker et al., 2010; Byrne, 2011). In particular, Kurihara et al. (2004) have observed a decline in egg production of *Acartia steueri* and *A. erythraea* at pH 6.8. Also, for *Palaemon pacificus* it was noted a decline in egg production at pH 7.6 (Kurihara et al., 2008). Hypercapnia reduces sperm swimming speed which can impair fertilization (Byrne, 2011). Despite these negative effects some species are quite robust and can support pH ranges of 7.4–7.6 without any consequences in fecundity (see Byrne, 2011; Whiteley, 2011).

The effects of LNG on *G. locusta* fecundity were quite different from those reported in the literature for fishes and other vertebrates. Following the effects of progestins described in the

literature it would be expected a decline or even a complete absence of reproduction under the exposure of increasing doses of LNG, however this was not observed for the gammarids. Despite the effects of progestins on reproduction of fishes and other vertebrates are quite well studied (Orlando and Ellestad, 2014; Kumar et al., 2015), for the invertebrates there is almost a complete gap of knowledge. A previous study that evaluated the effects of two estrogenic compounds (ethinylestradiol and 17- estradiol) and the progestin medroxyprogesterone on *Ceriodaphnia dubia*, also, did not find effects on its fecundity, even at concentrations in the range of mgL^{-1} (Jukosky et al., 2008). Other study from Kashian and Dodson (2004) also did not observe effects of estradiol on *D. magna* sex determination and reproduction at concentrations of 1, 10 and 100 $\mu g\ L^{-1}$. Based on these results it seems that the responses of invertebrates are quite different from those in aquatic vertebrates. According to the literature it is well known the negative effects of progestins on aquatic vertebrates' reproduction leading to a delay in the maturation of gonads, decline on female fish fecundity and consequently eggs production inhibition (Runnalls et al., 2013; Orlando and Ellestad, 2014; Kumar et al., 2015; Cardoso et al., 2017). We can hypothesise that a different response of the *G. locusta* to LNG can be related with several factors, such as: the discontinuity in the exposure to LNG or even the existence of different mechanisms of action of the progestin on the endocrine system of the amphipods. Anyway, it is relevant to recall that crustaceans express progesterone receptors in various organs, and that (at least) progesterone has a role in gonadal maturation (Ye et al., 2010; Wu et al., 2014).

Regarding the embryonic development, the temperature clearly had a significant impact on the speed of the process (as defined here). This is in accordance with the metabolic theory, so, as temperature increases, the rate of basic metabolic processes will also increase due to the kinetic effects of temperature on fundamental cellular processes (Brown et al., 2004). This can explain that under higher temperature the embryonic development of *G. locusta* was faster for the control treatments. However, the same was not true for those treatments exposed to LNG concentrations. So, it seems that the hormone inhibits somehow the stimulatory effect of the temperature, being the embryonic development similar to an ambient temperature scenario. Concluding, LNG did not negatively affect the embryonic development of *G. locusta* which is in accordance with previous studies developed in zebrafish with the progestin megestrol acetate (Han et al., 2014). However, for invertebrates no data exists and for fish the disruption potential varies with the progestin; e.g., LNG impacted on expression of embryonic development governing genes in the mosquitofish (Brockmeier et al., 2016).

Concluding, it seems that temperature and pCO_2 are the main driving factors that affect the fitness and reproduction of *G. locusta*, being the temperature the most dominant variable. Levonorgestrel did not reveal to have a relevant impact, particularly, at the reproduction level that would be one of the major topics of concern, however in certain occasions LNG modulated the effects of environmental drivers. Actually, there is a great lack of information concerning the combined effects of multiple stressors, especially including emergent compounds like the synthetic progestins. So, in the near future it is important to develop more research on these topics in order to better understand the functioning of coastal ecosystems and contribute for its better management and conservation.

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References

Appelhans, Y.S., Thomsen, J., Pansch, C., Melzner, F., Wahl, M., 2014. Sour times: seawater acidification effects on growth, feeding behaviour and acid-base status of *Asterias rubens* and *Carcinus maenas*. Mar. Ecol. Prog. Ser. 459, 85–97.

Boyd, P.W., Cornwall, C.E., Davison, A., Doney, S.C., Fourquez, M., Hurd, C.L., Lima, I.D., McMinn, A., 2016. Biological responses to environmental heterogeneity under future ocean conditions. Glob. Change Biol. 22, 2633–2650.

Brockmeier, E.K., Scott, P.D., Denslow, N.D., Leusch, F.D.L., 2016. Transcriptomic and physiological changes in Eastern mosquitofish (*Gambusia holbrooki*) after exposure to progestins and anti-progestagens. Aquat. Toxicol. 179, 8–17.

Brown, J.H., Gillooly, J.F., Allen, A.P., Savage, V.M., West, G.B., 2004. Toward a metabolic theory of ecology. Ecology 85, 1771–1789.

Byrne, M., 2011. Impact of ocean warming and ocean acidification on marine invertebrate life history stages. In: Gibson, R.N., Atkinson, R.J.A., Gordon, J.D.M. (Eds.), Oceanography and Marine Biology. CRC Press, Boca Raton, Florida, pp. 1–44.

Byrne, M., Przeslawski, R., 2013. Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. Integr. Comp. Biol. 53 (4), 582–596.

Cardoso, P.G., Rodrigues, D., Madureira, T.V., Oliveira, N., Rocha, M.J., Rocha, E., 2017. Warming modulates the effects of the endocrine disruptor progestin levonorgestrel on the zebrafish fitness, ovary maturation kinetics and reproduction success. Environ. Pollut. 229, 300–311.

Cardoso, P.G., Grilo, T.F., Dionisio, G., Aurélio, M., Lopes, A.R., Pereira, R., Pacheco, M., Rosa, R., 2017b. Short-term effects of increased temperature and lowered pH on a temperate grazer-seaweed interaction (*Littorina obtusata*/*Ascophyllum nodosum*). Estuar. Coast. Shelf Sci. 197, 35–44.

Cifoni, M., Galassi, D.M.P., Faraloni, C., Di Lorenzo, T., 2017. Test procedures for measuring the (sub)chronic effects of chemicals on the freshwater cyclopoid *Eucyclops serrulatus*. Chemosphere 173, 89–98.

Cornwall, C.E., Hurd, C.L., 2015. Experimental design in ocean acidification research: problems and solutions. ICES J. Mar. Sci. J. du Conseil 73 (3), 572–581.

Costa, F.O., Costa, M.H., 1999. Life history of the amphipod *Gammarus locusta* in the Sado estuary (Portugal). Acta Oecol. 20 (4), 305–314.

Costa, F.O., Costa, M.H., 2000. Review of the ecology of *Gammarus locusta* (L.). Pol. Arch. Hydrobiol. 48, 541–559.

Costa, F.O., Neuparth, T., Correia, A.D., Costa, M.H., 2005. Multi-level assessment of chronic toxicity of estuarine sediments with the amphipod *Gammarus locusta*: II. Organism and population-level endpoints. Mar. Environ. Res. 60, 93–110.

de Alda, M.J.L., Gil, A., Paz, E., Barcelo, D., 2002. Occurrence and analysis of estrogens and progestogens in river sediments by liquid chromatography-electrospray mass spectrometry. Analyst 127, 1299–1304.

Di Lorenzo, T., Di Marzio, W.D., Cifoni, M., Fiasca, B., Baratti, M., Sáenz, M.E., Galassi, D.M.P., 2015. Temperature effect on the sensitivity of the copepod *Eucyclops serrulatus* (Crustacea, Copepoda, Cyclopoida) to agricultural pollutants in the hyporheic zone. Curr. Zool. 61 (4), 629–640.

Elisio, M., Chalde, T., Miranda, L.A., 2012. Effects of short periods of warm water fluctuations on reproductive endocrine axis of the pejerrey (*Odontesthes bonariensis*) spawning. Comp. Biochem. Physiol., part A 163, 47–55.

Enin, U., 1994. Length-weight parameters and condition factor of two West African prawns. Rev. Hydrobiol. Trop. 27 (2), 121–127.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environ. Int. 84, 115–130.

Gillet, C., Breton, B., Mikolajczyk, T., Bodinier, P., Fostier, A., 2011. Disruption of the secretion and action of 17,20 β-dihydroxy-4-pregnen-3-one in response to a rise in temperature in the Arctic charr, *Salvelinus alpinus*. Consequences on oocyte maturation and ovulation. General Comp. Endocrinol. 172, 392–399.

Gutow, L., Rahman, M.M., Bartl, K., Saborowski, R., Bartsch, I., Wiencke, C., 2014. Ocean acidification affects growth but not nutritional quality of the seaweed *Fucus vesiculosus* (Phaeophyceae, Fucales). J. Exp. Mar. Biol. Ecol. 453, 84–90.

Han, J., Wang, Q., Wang, X., Li, Y., Wen, S., Liu, S., Ying, G., Guo, Y., Zhou, B., 2014. The synthetic progestin megestrol acetate adversely affects zebrafish reproduction. Aquat. Toxicol. 150, 66–72.

Harvey, B.P., Gwynn-Jones, D., Moore, P.J., 2013. Meta-analysis reveals complex marine biological responses to the interactive effects of ocean acidification and warming. Ecol. Evol. 3, 1016–1030.

Hauton, C., Tyrrell, T., Williams, J., 2009. The subtle effects of sea water acidification on the amphipod *Gammarus locusta*. Biogeosciences 6, 1479–1489.
Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., et al., 2013. Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. Environ. Toxicol. Chem. 32 (1), 32–48.
Hutchinson, T.H., Shillabeer, N., Winter, M.J., Pickford, D.B., 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. Aquat. Toxicol. 76, 69–92.
Jacobson, T., Prevodnik, A., Sundelin, B., 2008. Combined effects of temperature and a pesticide on the Baltic amphipod *Monoporeia affinis*. Aquat. Biol. 1, 269–276.
Jukosky, J.A., Watzin, M.C., Leiter, J.C., 2008. Elevated concentrations of ethinylestradiol, 17 β-Estradiol and medroxyprogesterone have little effect on reproduction and survival of *Ceriodaphnia dubia*. Bull. Environ. Contam. Toxicol. 81, 230–235.
Kay, P., Hughes, S.R., Ault, J.R., Ashcroft, A.E., Brown, L.E., 2017. Widespread, routine occurrence of pharmaceuticals in sewage effluent, combined sewer overflows and receiving waters. Environ. Pollut. 220, 1447–1455.
Kashian, D.R., Dodson, S.I., 2004. Effects of vertebrate hormones on development and sex determination in *Daphnia magna*. Environ. Toxicol. Chem. 23, 1282–1288.
Kroeker, K.J., Kordas, R.L., Crim, R.N., Singh, G.G., 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol. Lett. 13 (11), 1419–1434.
Kroeker, K.J., Kordas, R.L., Crim, R., et al., 2013. Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. Glob. Change Biol. 19, 1884–1896.
Kroeker, K.J., Sanford, E., Jellison, B.M., Gaylord, B., 2014. Predicting the effects of ocean acidification on predator-prey interactions: a conceptual framework based on coastal molluscs. Biol. Bull. 226, 211–222.
Kumar, V., Johnson, A.C., Trubiroha, A., Tumová, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kroupová, K., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environ. Sci. Technol. 49 (5), 2625–2638.
Kummerer, K., 2009. The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. J. Environ. Manag. 90 (8), 2354–2366.
Kurihara, H., Shimode, S., Shirayama, Y., 2004. Effects of raised CO_2 concentration on the egg production rate and early development of two marine copepods (*Acartia steueri* and *Acartia erythraea*). Mar. Pollut. Bull. 49, 721–727.
Kurihara, H., Matsui, M., Furukawa, H., Hayashi, M., Ishimatsu, A., 2008. Long-term effects of predicted future seawater CO_2 conditions on the survival and growth of the marine shrimp *Palaemon pacificus*. J. Exp. Mar. Biol. Ecol. 367, 41–46.
Lenth, R.V., 2016. Least-Squares Means: the R package lsmeans. J. Stat. Softw. 69 (1), 1–33.
Lopez, L.M., Edelman, A., Chen, M., Otterness, C., Trussell, J., Helmerhorst, 2013. Progestin-only contraceptives: effects on weight. Cochrane Database Syst. Rev. 7, CD008815.
Martin, D., Elliott-Sale, K., 2016. A perspective on current research investigating the effects of hormonal contraceptives on determinants of female athlete performance. Rev. Bras. Educ. Física Esporte 30.
Maranhão, P., Marques, J.C., 2003. The influence of temperature and salinity on the duration of embryonic development, fecundity and growth of the amphipod *Echinogammarus marinus* Leach (Gammaridae). Acta Oecol. 24 (1), 5–13.
Meehl, G.A., Stocker, T.F., Collins, W.D., Friedlingstein, P., Gaye, A.T., Gregory, J.M., et al., 2007. Global climate projections. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., et al. (Eds.), Climate Change 2007: the Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.
Miranda, L.A., Chalde, T., Elisio, M., Strussmann, C.A., 2013. Effects of global warming on fish reproductive endocrine axis, with special emphasis in pejerrey *Odontesthes bonariensis*. General Comp. Endocrinol. 192, 45–54.
Moreira, C.A., Ferreira De Carvalho, A.A., Campos, J.V., 2015. Comparing the response of the brown shrimp *Crangon crangon* (linnaeus, 1758) to prolonged deprivation of food in two seasons. J. Shellfish Res. 34 (2), 521–529.
Neuparth, T., Costa, F.O., Costa, M.H., 2002. Effects of temperature and salinity on life history of the marine amphipod *Gammarus locusta*. Implications for ecotoxicological testing. Ecotoxicology 11 (1), 61–73.
Neuparth, T., Martins, C., Santos, C.B.D.L., Costa, M.H., Martins, I., Costa, P.M., Santos, M.M., 2014. Hypocholesterolaemic pharmaceutical simvastatin disrupts reproduction and population growth of the amphipod *Gammarus locusta* at the ng/L range. Aquat. Toxicol. 155, 337–347.
Noyes, P.D., Lema, S.C., 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. Curr. Zool. 61 (4), 669–689.
O'Connor, M.I., 2009. Warming strengthens an herbivore–plant interaction. Ecology 90, 388–398.
Orlando, E.F., Ellestad, L.E., 2014. Sources, concentrations and exposure effects of environmental gestagens on fish and other aquatic wildlife with an emphasis on reproduction. General Comp. Endocrinol. 203, 241–249.
Overturf, M.D., Overturf, C.L., Carty, D.R., Hala, C., Huggett, D.B., 2014. Levonorgestrel exposure to fathed minnows (*Pimephales promelas*) alters survival, growth, steroidogenic gene expression and hormone production. Aquat. Toxicol. 148, 152–161.
Overturf, D.O., Huggett, D.B., 2015. Responses to various exposure durations of levonorgestrel during early-life stages of fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 161, 33–40.
Pankhurst and Munday, 2011. Effects of climate change on fish reproduction and early life history stages. Mar. Freshw. Res. 62, 1015–1026.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Core Team, R., 2017. n*lme: Linear and Nonlinear Mixed Effects Models*. R Package Version 3.1-131. https://CRAN.R-project.org/package=nlme.
Poore, A.G.B., Graba-Landry, A., Favret, M., Brennand, H.S., Byrne, M., Dworjanyn, S.A., 2013. Direct and indirect effects of ocean acidification and warming on a marine plant–herbivore interaction. Oecologia 173, 1113–1124.
Portner, H.O., Farrell, A.P., 2008. Physiology and climate change. Science 322, 690–692.
R Core Team, 2016. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. Bull. Fish. Res. Board Can. 191, 1–382.
Rosa, R., Baptista, M., Lopes, V.M., Pegado, M.R., Ricardo Paula, J., Trübenbach, K., Leal, M.C., Calado, R., Repolho, T., 2014. Early-life exposure to climate change impairs tropical shark survival. Proc. R. Soc. Lond. B Biol. Sci. 281, 1–7.
Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environ. Sci. Technol. 47, 2077–2084.
Sampaio, E., Rodil, I.F., Vaz-Pinto, F., Fernandez, A., Arenas, F., 2017. Interaction strength between different grazers and macroalgae mediated by ocean acidification over warming gradients. Mar. Environ. Res. 125, 25–33.
Sissom, E.K., Reinhardt, C.D., Johnson, B.J., 2006. Melengestrol acetate alters muscle cell proliferation in heifers and steers. J. Animal Sci. 84, 2950–2958.
Talmage, S.C., Gobler, C.J., 2009. The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (*Mercenaria mercenaria*), bay scallops (*Argopecten irradians*), and Eastern oysters (*Crassostrea virginica*). J. Limnol. Oceanogr. 54, 2072–2080.
Vulliet, E., Baugros, J.B., Flament-Waton, M.M., Grenier-Loustalot, M.F., 2007. Analytical methods for the determination of selected steroid sex hormones and corticosteroids in wastewater. Anal. Bioanal. Chem. 387, 2143–2151.
Vulliet, E., Wiest, L., Baudot, R., Grenier-Loustalot, M.F., 2008. Multi-residue analysis of steroids at sub-ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. J. Chromatogr. 1210, 84–91.
Whiteley, N.M., 2011. Physiological and ecological responses of crustaceans to ocean acidification. Mar. Ecol. Prog. Ser. 430, 257–271.
Wu, X., Chen, H., Liu, Z., Cheng, Y., 2014. Immunorecognition and distribution of progesterone receptors in the Chinese mitten crab *Eriocheir sinensis* during ovarian development. J. Shellfish Res. 33, 35–43.
Ye, H., Song, P., Ma, J., Huang, H., Wang, G., 2010. Changes in progesterone levels and distribution of progesterone receptor during vitellogenesis in the female mud crab *Scylla paramamosain*. Mar. Freshw. Behav. Physiol. 43, 25–35.